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Article Addendum

Probing allelochemical biosynthesis in sorghum root hairs

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Allelopathic interaction between plants is thought to involve the release of phytotoxic allelochemicals by one species, thus inhibiting the growth of neighboring species in competition for limited resources. Sorgoleone represents one of the more potent allelochemicals characterized to date, and its prolific production in root hair cells of *Sorghum spp.* has made the investigation of its biosynthetic pathway ideally-suited for functional genomics investigations. Through the use of a recently-released EST data set generated from isolated *Sorghum bicolor* root hair cells, significant inroads have been made toward the identification of genes and the corresponding enzymes involved in the biosynthesis of this compound in root hairs. Here we provide additional information concerning our recent report on the identification of a 5-*n*-alk(en)ylresorcinol utilizing *O*-methyltransferase, as well as other key enzymes likely to participate in the biosynthesis of this important allelochemical.

Introduction

Allelopathic interactions have been proposed to have profound effects on the evolution of natural ecosystems by conferring a competitive advantage to allelochemical-producing species, or species resistant to interference from a given allelochemical.[1,2] In addition, allelochemical release by certain crop species such as barley, rye and sorghum is thought to play a significant role in their utility as weed suppressants when used as cover crops.[3,4] Certain sorghum species such as Sudangrass (*Sorghum sudanense*) can reportedly produce largely weed-free monocultures without the use of synthetic herbicides.[5]

Current evidence suggests that a highly bioactive benzoquinone synthesized in root hair cells of *Sorghum spp.* termed sorgoleone (2-hydroxy-5-methoxy-3-[(*Z*,*Z*)-8',11',14'-pentadecatriene]-*p*-benzoquinone—Fig. 1) may represent the principal chemical constituent responsible for conferring allelopathic properties.[6-8] Studies performed in vitro with purified sorgoleone have demonstrated its effectiveness as a broad-spectrum plant growth inhibitor active against many agronomically important monocot and dicot weed species at micromolar concentrations. In fact, this property, coupled with its prolonged half-life in soil and potentially complex mode of action, has led to the suggestion that sorgoleone could be developed as a useful natural product alternative to synthetic herbicides.[6-12] Interestingly, within the *Poaceae* (grass) family, sorgoleone biosynthesis may be restricted to members of the genus *Sorghum*, as a limited survey involving root samples collected from 17 different *Poaceae* accessions failed to detect sorgoleone production even in the most closely-related *Panicoideae* sub-family members (Fig. 2A).

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Use of Functional Genomics to Explore Sorgoleone Biosynthesis

As mentioned, sorgoleone biosynthesis appears to occur exclusively in root hair cells, which in sorghum appear as cytoplasmically dense cells filled with numerous osmiophilic deposits presumably associated with the rhizosecretion of sorgoleone, which can constitute as much as 85% of the exudate dry weight in some cultivars.[8,13] The cell-specific localization and prolific output of the sorgoleone biosynthetic pathway rendered the use of expressed sequence tag (EST) analysis the obvious method of choice in our efforts to isolate genes encoding sorgoleone biosynthetic enzymes.[14] Labeling studies performed by Fate & Lynn,[15] first demonstrated that biosynthesis proceeds through the action of an alkylresorcinol synthase (ARS), a novel type III polyketide synthase activity utilizing fatty acyl-CoA starter units (Fig. 1). Subsequently, both the predicted 5-*n*-pentadecatrienyl resorcinol as well as a 3-methyl ether derivative of this compound were identified in sorghum root extracts, indicating that dihydroxylation of the resorcinol ring is preceded by *O*-methylation at the 3-hydroxyl position.[14,16] The resulting chemically unstable hydroquinone rapidly oxidizes to the bioactive sorgoleone benzoquinone once released into the rhizosphere, where it may persist in soil for extended periods.[7,8,17] De novo synthesis of sorgoleone from available palmitoleoyl-CoA, would therefore likely require, at a minimum, the participation of a Δ^{12} and Δ^{15} fatty acid desaturase (DES), ARS, a 3-*O*-methyltransferase (OMT), and a cytochrome P450 (Fig. 1).

To obtain candidate sequences for each of these proposed enzyme classes, 5,468 quality 5'-sequenced ESTs were generated from a primary phagemid cDNA library prepared from isolated root hair cells, and all sequence data has been recently deposited in GenBank.

Importantly, the *S. bicolor* genotype BTx623 was used as the source of the root hair tissues, therefore the ESTs can be directly compared to the emergent *S. bicolor* whole-genome sequence which will also be derived from BTx623.[18] Clustering and annotation of the root hair data set revealed 7 unique DES-like sequences, 5 polyketide synthase (PKS)-like sequences, 12 OMT-like sequences, and 11 P450-like sequences, thus all of the required enzyme classes proposed for sorgoleone biosynthesis were represented. Using *Saccharomyces cerevisiae* as a heterologous host, we have recently demonstrated the ability of two fatty acid desaturases (SbDES2, SbDES3) identified from the root hair EST data set to catalyze the formation of the unusual 16:3$\Delta^{9,12,15}$ fatty acyl-CoA biosynthetic precursor (Fig. 1) in vivo, strongly suggesting they perform this same function in planta.[19] The functional characterization of an OMT proposed to perform the 3-*O*-methylation of the 5-*n*-pentadecatrienyl resorcinol intermediate, designated SbOMT3, is described in our recent report which also provides secondary analyses of the root hair EST data.[14] Two PKS-like sequences identified from the root hair ESTs, tentatively designated *SbARS1* and *SbARS2* (Fig. 1), have also recently been shown to catalyze the formation of 5-*n*-alk(en)ylresorcinols utilizing an array of saturated and unsaturated fatty acyl-CoA starter units, thus representing the first ARS-type enzymes cloned from higher plants (manuscript in preparation).

Potential Genetic Redundancy of the 3-*O*-Methylation Function

Recombinant enzyme studies involving three of the OMT-like enzymes (SbOMT1, SbOMT2, SbOMT3—Fig. 2B) identified among the root hair ESTs were described in our recent report,[14] and SbOMT3 demonstrated a marked preference for alkylresorcinolic substrates among a panel of benzene derivatives tested in that study. As mentioned, in total 12 unique sequences resembling OMTs were identified in root hairs, and expression patterns in different sorghum tissues were determined for all of these (Fig. 2B). In addition to SbOMT1, SbOMT2, and SbOMT3, two of the remaining 9 OMT-like contig sequences (2_54 and 0_1308; Fig. 2B) were found to be expressed predominantly in root hairs, and were also among the most highly expressed sequences in this cell type.[14] Using the partial sequence data available from contig 2_54, a full length open reading encoding an approximately 40.7 kDa OMT-like protein could be deduced when aligned with the available sorghum genome draft sequence. Interestingly 2_54 shares 93% amino acid identity with SbOMT3, and all of critical active site residues proposed for SbOMT3 (based on molecular modeling studies)[14] appear to be conserved, suggesting the possibility that this putative OMT could have similar catalytic properties. While the current sorghum genome assembly (Sorbi0; http://www.phytozome.net/sorghum) is a preliminary draft based on a partial data set, available evidence indicates that *SbOMT3* is actually represented as three closely-linked copies, one of which is an apparent 5'-truncated pseudogene (Fig. 2C). Of the three predicted OMT-like genes residing within an approximately 30 kb chromosomal region, Sbi_0.22025 and Sbi_0.22026 share 100% and 99% nucleotide identity with the *SbOMT3* open reading frame predicted from the root hair ESTs, respectively. The truncated copy corresponds to Sbi_0.22025 (Fig. 2C), and lacks the first approximately 500 bp of the OMT open reading frame, with the remainder sharing 98% identity with *SbOMT3*. Extensive sequence identity also exists within the regions flanking the reading

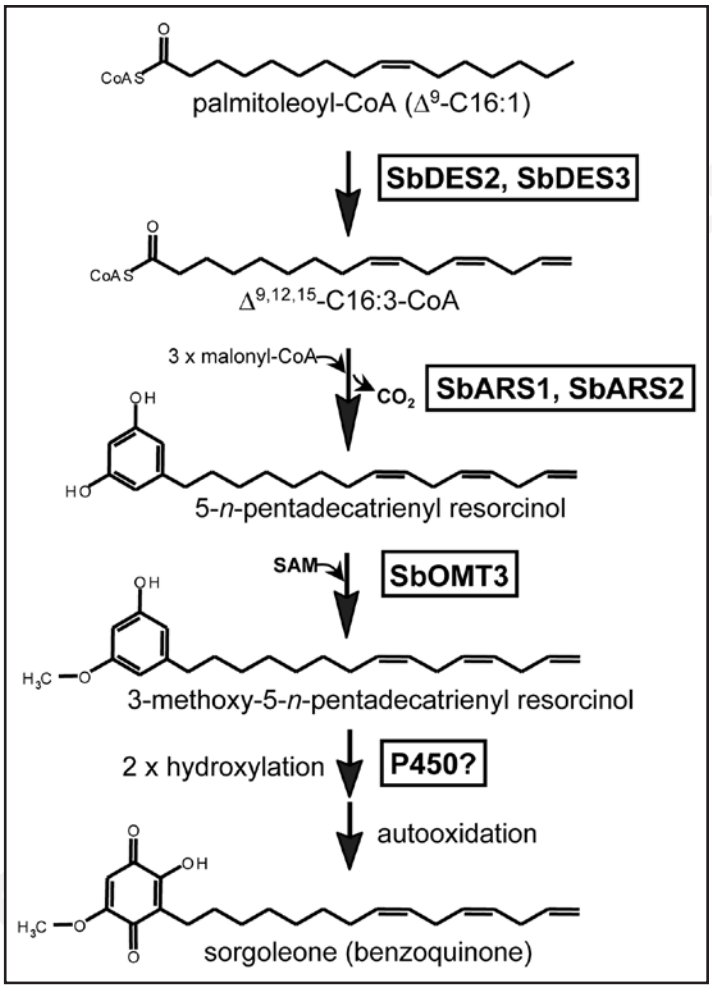

Figure 1.The current view of the sorgoleone biosynthetic pathway is shown.

frames of Sbi_0.22024, Sbi_0.22025 and Sbi_0.22026, suggesting the occurrence of a recent gene duplication event. The predicted gene corresponding to root hair contig 2_54 resides on an entirely different Sorbi0 super-cluster and may not be closely-linked to *SbOMT3* (Sbi_0.22025). While the high degree of sequence identity shared between the 5' and 3' flanking regions of *SbOMT3* and Sbi_0.22026 would suggest identical expression patterns for the two genes, even minor changes within critical motifs could lead to significant alterations in promoter activity, potentially providing the basis for functional divergence. Examining the substrate specificity of putative OMT 2_54, and the potential role played by Sbi_0.22026 in sorgoleone biosynthesis represent just two out of many intriguing questions awaiting further exploration.

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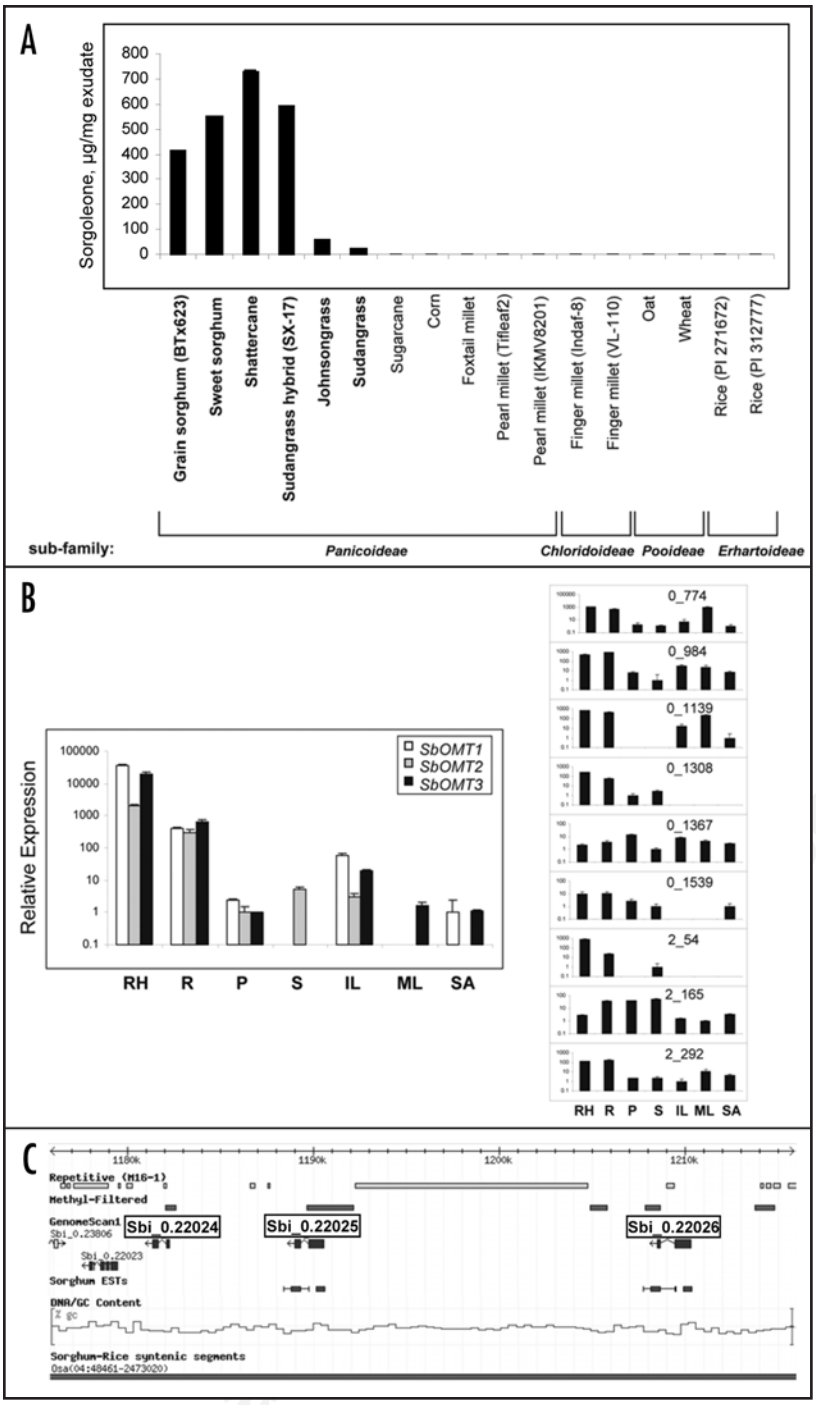

Figure 2. Survey of sorgoleone production in grasses, expression patterns of *O*-methyltransferase-like sequences identified in root hairs, and genomic organization of *SbOMT3*-like copies are shown. (A) Sorgoleone contents were determined in root extracts prepared from representative *Poaceae* accessions by GC-MS analysis;[14,20] including grain sorghum (*S. bicolor* 'BTx623'), sweet sorghum (*S. bicolor* 'Della'), shattercane (*S. bicolor* ssp. *drummondii*), sorghum-sudangrass hybrid (*S. bicolor* x *sudanense* 'SX-17'), johnsongrass (*S. halapense* L. Pres.), sudangrass (*S. sudanense* 'Excel'), sugarcane (*Saccharum* spp. hybrid 'HoCP 91-555'), corn (*Zea mays* 'Merit'), foxtail millet (*Setaria italica* 'Golden German'), pearl millet (*Pennisetum glaucum* 'Tifleaf2' and 'IKMV8201'), finger millet (*Eleusine coracana* 'Indaf-8' and 'VL-110'), Oat (*Avena sativa* 'Bob'), wheat (*Triticum aestivum* 'Coker 9553') and two allelopathic rice varieties (*Oryza sativa* 'PI271672' and 'PI312777')[21]. Averages obtained from replicate samples are shown, and associated *Poaceae* subfamilies are indicated by brackets as assigned by Kellogg.[22] (B) Relative expression patterns in different sorghum tissues for OMT-like sequences identified in root hairs were determined by quantitative real-time RT-PCR. *SbOMT1-3* graph (shown at left) was adapted from Baerson et al.;[14] contig I.D. numbers for the remaining 9 OMT-like sequences identified (shown at right) are provided within each graph. RH, root hair; R, total root; P, panicle; S, stem; IL, immature leaf; ML, mature leaf; SA, shoot apex. (C) Sorghum Genome Browser return (http://www.phytozome.net) is shown indicating three closely-linked *SbOMT3*-like copies within the *S. bicolor* ('BTx623') genome (corresponding to GenomeScan1 predicted ORFs Sbi_0.22024, Sbi_0.22025 and Sbi_0.22026 shown in figure).

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